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13. ABSTRACT (Maximum 200 Words) <p>The overall goal of this proposal were to test the hypothesis that eosinophils promote DNA oxidative damage in breast carcinoma. DNA oxidative damage is linked to mutation, transformation and cancer development and eosinophil peroxidase (EPO), a hemoprotein secreted from eosinophils, is present in the majority of breast cancer biopsies. Our initial aim was to determine whether EPO promotes oxidative damage of cellular DNA through formation of mutagenic hydroxyl radical (*OH)-generated bases. We have shown (Biochemistry, (2000) 39:5474) that activated leukocytes can oxidatively damage DNA, RNA and the nucleotide pool through halide-dependent formation of *OH. *OH-dependent damage of DNA was quantified by monitoring the content of 8-hydroxyguanine (8OHG), an established *OH-specific DNA oxidation product that is mutagenic and implicated in breast cancer development and progression to metastatic disease. To test the hypothesis that EPO promotes DNA oxidative damage in human breast carcinoma, we have identified a family of novel brominated DNA oxidation products. These may serve as "molecular fingerprints" for DNA damage by the EPO pathway of eosinophils. Results of our studies identifying brominated bases by mass spectrometry were recently published (Biochemistry, (2001) 40:2041-2051). Most recently we have shown that markers of nitric oxide-derived oxidants, species formed by eosinophils, are correlated microvascular density in breast carcinoma (Breast Cancer Research and Treatment (2002) 74:271-278). We are presently performing studies aimed at quantifying these EPO-specific brominated bases in a well-characterized repository of breast carcinoma and microscopically normal breast tissue specimens.</p>				
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Introduction

The contribution of oxidative processes to DNA damage and carcinogenesis is now widely accepted (1-13). However, the precise chemical pathways involved remain unclear. One method that has contributed significantly to the conclusion that DNA undergoes oxidative damage is the detection of multiple distinct stable markers of free-radical reactions *in vivo*. For example, markers of $\cdot\text{OH}$ -, reactive nitrogen species- and aldehyde-dependent damage of DNA have been used to implicate specific chemical mechanisms of DNA damage *in vivo* (14-20). Eosinophil recruitment is characteristic of many cancers and numerous chronic parasitic infections are associated with a dramatic increase in risk for development of cancer (21-25), yet the potential role of eosinophils in mediating DNA damage and cancer development has not received attention. Studies of hormone-responsive tissues (e.g. breast and uterus) suggest that eosinophil migration occurs in response to estrogen administration. (26-29), and elevated peroxidase activity is found in human breast cancer (30-31). In our proposal we hypothesized that eosinophils may contribute to DNA oxidative damage and cancer. The vast majority of breast cancers stain positively for eosinophil peroxidase (EPO), a hemoprotein specific for eosinophils. EPO catalyzes oxidation reactions as part of its normal role in host defenses. We hypothesize that the presence of EPO in breast cancer tissues suggests that EPO could contribute to oxidative modification of DNA and the nucleotide pool, and thus potentially play a role in the disease process. We proposed to identify chemical mechanisms through which eosinophils might contribute to DNA oxidative damage. We also proposed to define novel specific chemical markers indicative of eosinophil-mediated DNA damage *in vivo*. Finally, we proposed to use mass spectrometry to establish whether these markers are enriched in breast cancer specimens - thus identifying for the first time a pathogenic role for eosinophils in cancer development.

Body

Task 1. To determine whether eosinophil peroxidase promotes oxidative damage of cellular DNA through formation of mutagenic hydroxyl radical-generated bases.

All of the sub-goals as outlined in Task 1 of the approved Statement of Work were achieved in prior a (1999-2000) reporting interval. Detailed descriptions of the methodology and results were provided with that preceding annual report. A copy of a publication that is based upon this work was provided in last year's report as Appendix item 1:

Z. Shen, W. Wu and S. L. Hazen "Activated leukocytes oxidatively damage DNA, RNA and the nucleotide pool through halide-dependent formation of hydroxyl radical. *Biochemistry* (2000), 39:5474-5482.

Task 2. To test the hypothesis that eosinophil peroxidase promotes DNA oxidative damage in human breast carcinoma

- a. *Synthesize, HPLC purify and confirm structures of brominated bases by ESI/MS, GC/MS and multinuclear NMR*
- b. *Synthesize, HPLC purify and confirm structure of stable isotope-labeled brominated bases by GC/MS*
- c. *optimize DNA hydrolysis/digestion/sample work-up for quantification of brominated bases in DNA exposed to HOBr*
- d. *perform experiments to evaluate Br-dG and Br-dC formation in calf thymus DNA exposed to HOBr, and then the EPO-H₂O₂-Br- system*

All of the sub-goals as outlined above in Task 2 of the approved Statement of Work were achieved and reported in last years (2000-2001) reporting interval. Detailed descriptions of the methodology

and results are enumerated in a recent publication that is based upon this work. A copy of the manuscript was provided as Appendix item 2 in last year's report:

Shen, Z., Mitra, S.N., Wu, W., Chen, Y., Yang, Y., Qin, J and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," *Biochemistry* (2001) 40:2041-2051.

The results of these studies were also presented at 2 international meetings. A copy of the abstracts was provided in last year's report as Appendix items 3 and 4:

a) Shen, Z., and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," Abstract 263, of 7th Annual Meeting of the Oxygen Society, Nov 16-20, 2000. *Free Radical Biology & Medicine*, (2000) Vol 29, Supplement I, S85.

b) Shen, Z., and Hazen, S.L. "Oxidative damage of DNA by peroxidase-mediated bromination," Oral Presentation, June (2000), American Society of Mass Spectrometry Meeting, Long Beach, CA.

Progress for current reporting interval:

e. *perform experiments to assess ability of EPO to promote Br-dG and Br-dC formation in cell culture model as well as the factors influencing DNA damage by this pathway*

We have performed studies with cultured cells (HA1 cells) exposed to the EPO-H₂O₂-Br⁻ system of activate eosinophils. The in vitro models employed are the same as those developed and utilized for demonstration of [•]OH formation by EPO, as assessed by 8OHG generation in cytosolic bases and DNA of target cells (*Biochem.* (2000), 39:5474-5482). We have observed bromination of bases in the free nucleoside pool, as well as bromination of RNA bases. However, we have thus far been unable to demonstrate that bromination of bases within double-stranded DNA occurs within intact cells as targets. Only following extremely harsh conditions that promote cell death and lysis do we observe DNA bromination. Current studies are now being aimed at determining if brominated bases in tissue culture media can be taken up and incorporated into DNA. Such a process might serve as an alternative mechanisms for promoting DNA modifications within rapidly expanding cells.

f. *quantify brominated bases (Br-dG and Br-dC) in DNA recovered from normal, dysplastic, cancerous and metastatic breast tissue*

This work is still in progress. We applied our developed stable isotope dilution LC/ESI/MS/MS methods to analyze breast biopsy specimens from normal and cancerous specimens, and could detect no significant levels of the EPO-specific modifications. We think the current data supports the notion that brominated bases are specific markers for DNA damage at sites of eosinophilic inflammation - but there does not exist a significant enough degree of inflammation in most breast cancers to observe these specific markers.

We have recently shown that eosinophils serve as a major source of nitric oxide derived oxidants (32). These studies were based upon our earlier demonstration that purified human EPO preferentially generated nitrating oxidants under physiological conditions. Therefore, in new studies, we sought to determine whether we could find evidence for NO-derived oxidants as potential participants in damage of targets within breast cancers. Using a combination of histopathological analyses and mass spectrometry-based quantification of nitrotyrosine, a marker of protein damage by NO-derived oxidants, we recently found an association between nitrotyrosine

levels and the degree of microvascular density within human breast cancers. These results have recently been accepted and published.

Samoszuk, M., Brennan, M.L., To, V., Leonor, L., Zheng, L., Fu, X., and Hazen, S.L. "Association between nitrotyrosine levels and microvascular density in human breast cancer." *Breast Cancer Research and Treatment* (2002) 74:271-278.

Key Research Accomplishments

- 1) The leukocyte peroxidases EPO and MPO generate halogenating oxidants that combine with superoxide to form hydroxyl radical like species capable of damaging DNA.
- 2) Exposure of cells to an extracellular source of peroxidase-generated reactive halogen promotes hydroxylation of DNA, RNA and the nucleotide pool in the presence of superoxide. This results in formation of mutagenic bases and DNA damage characteristic of that observed in breast cancer and progression to metastatic disease.
- 3) Exposure of free nucleotides and DNA to either HOBr or the EPO-H₂O₂-Br⁻ system results in bromination of DNA bases. Several distinct brominated bases are known to be mutagenic.
- 4) The structures of the halogenated nucleobases have been defined.
- 5) Stable isotope dilution mass spectrometry-based methods have been developed to quantify the content of these novel molecular markers.
- 6) Insights into the chemical mechanisms accounting for nucleotide bromination and the structural/steric requirements for bromination of free nucleobases vs double stranded DNA have been defined.
- 7) Insights into the feasibility of nucleotide, RNA and DNA oxidation by eosinophil-generated brominating oxidants.
- 8) Lower limits of detection for brominated nucleobases in breast cancer tissues have been determined.
- 9) A correlation between breast cancer tissue levels of nitrotyrosine and microvascular density of cancer tissues has been noted. This suggests that nitric oxide-derived oxidants may participate in tissue invasion and angiogenesis within breast carcinoma.

Reportable outcomes (Bibliography)

Manuscripts:

Shen, Z., Wu, and Hazen, S.L. "Activated leukocytes oxidatively damage DNA, RNA and the nucleotide pool through halide-dependent formation of hydroxyl radical. *Biochemistry* (2000), 39:5474-5482.

Shen, Z., Mitra, S.N., Wu, W., Chen, Y., Yang, Y., Qin, J and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," *Biochemistry* (2001) 40:2041-2051.

Samoszuk, M., Brennan, M.L., To, V., Leonor, L., Zheng, L., Fu, X., and Hazen, S.L. "Association between nitrotyrosine levels and microvascular density in human breast cancer." *Breast Cancer Research and Treatment* (2002) 74:271-278.

Abstracts and presentations

Shen, Z., and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," Abstract 263, of 7th Annual Meeting of the Oxygen Society, Nov 16-20, 2000. *Free Radical Biology & Medicine*, (2000) Vol 29, Supplement I, S85.

Shen, Z., and Hazen, S.L. "Oxidative damage of DNA by peroxidase-mediated bromination," Oral Presentation, June (2000), American Society of Mass Spectrometry Meeting, Long Beach, CA. #755.

patents - None

degrees obtained - None

development of tissue repositories/cell lines - None

informatics - None

funding applied for based upon work - None

employment/research opportunities applied for/received - None

Conclusions

The contribution of oxidative processes to carcinogenesis is now widely accepted. Much progress in this area involves use of stable markers of free-radical reactions to identify specific chemical mechanisms of DNA damage *in vivo*. For example, hydroxylated, nitrated, aldehyde-modified, and chlorinated bases have been characterized and used to determine mechanisms of DNA, RNA and nucleotide damage *in vitro* and *in vivo*. However, the potential role of brominating oxidants in DNA damage and cancer development has not received much attention. The results of the present studies suggest that formation of reactive brominating species by the EPO-H₂O₂-Br⁻ system of eosinophils may be one pathway these cells contribute to oxidative modification of the nucleotide pool. Recent studies identify brominating oxidants as a distinct class of oxidants formed following eosinophil activation *in vivo*. Moreover, numerous cancers are notable for a significant eosinophilic infiltration in the cancerous tissues. The specific brominated bases identified may thus serve as markers for future studies aimed at determining the potential role of brominating oxidants in oxidative damage *in vivo*.

Based upon results from the present report and recent published studies, we have generated a model of potential pathways through which brominating oxidants may contribute to oxidative modification of free bases, RNA and DNA. Upon activation, the NADPH oxidase complex of eosinophils forms O₂^{•-}, which both spontaneously and enzymatically dismutates to form H₂O₂. Concomitantly, eosinophil activation leads to the secretion of EPO into the extracellular compartment. In the presence of plasma levels of Br⁻, EPO generates brominating oxidants like HOBr, which can directly brominate free nucleotides and DNA forming stable mono-brominated adducts. Identification of brominated adducts of each free base was confirmed by HPLC with on-line ESI-MS analysis. In the case of brominated purine bases, structural characterization as the 8-bromo-substituted analogs of adenine and guanine was further confirmed by NMR and tandem mass spectrometry. EPO-generated HOBr can also react with O₂^{•-} to form a [•]OH-like oxidant. The content of 8-hydroxyguanine, a marker of [•]OH-dependent DNA damage, was recently shown to significantly increase in DNA, RNA and the nucleotide pool of cells exposed to a hypohalous acid generating system and enhanced intracellular O₂^{•-}.

One critical question that we have not yet been able to unequivocally resolved is whether bromination of purine and pyrimidine targets takes place *in vivo*. This will have to serve as the focus of research efforts in the future. While our initial goal was to analyze breast cancer specimens, it seems prudent to modify this for the future, and also look at other sites of inflammation where eosinophil-mediated inflammation and cancer development occur.

We have obtained access to biopsy specimens from individuals with schistosomiasis, a chronic parasitic infection that results in dramatic increases in cancer. We are currently performing studies aimed at detection of brominated DNA and/or nucleotides from isolated schistosomes. We feel this would serve as *in vivo* "proof of concept" for the central idea of the proposal. Clearly, the probability of a brominating oxidant diffusing through a gauntlet of cytosolic scavengers unscathed before reaching a nuclear DNA base as its ultimate target will be a low probability event (as are all DNA oxidation events). However, it should also be recognized that the oxidation event does not have to take place inside the cell nucleus, but may occur either within the cytosol (i.e. the nucleotide pool) or even within the extracellular compartment. Parasitic infections are accompanied by increased cell death and lysis at the site of inflammation. Moreover, brominated bases are reported to be taken up and incorporated into DNA and RNA of cultured mammalian cells. Current efforts are aimed at assessing the incorporation of brominated bases into DNA of rapidly dividing cultured cells.

Though mammalian cells are equipped with numerous surveillance mechanisms for removal of modified bases from the nucleotide pool, the fidelity of these systems is not absolute. Indeed, exposing cultured cells to brominated bases in the media results in sister chromatid exchanges and mutation. We have recently demonstrated that bromination of extracellular targets (protein tyrosine residues) by activated eosinophils occurs *in vivo* at sites of inflammation. The ability of certain free nucleobases to undergo bromination at neutral pH suggests that similar events may occur *in vivo*. Thus, in the setting of a chronic parasitic infection where decades of eosinophil-mediated inflammatory injury can occur, bromination of extracellular or cytosolic nucleobases may occur.

In summary, the results obtained during the conduct of the present research proposal suggest that specific brominated DNA bases may serve as novel and specific markers for monitoring oxidative damage of DNA and the nucleotide pool by brominating oxidants. The detection of brominated bases in eosinophil-rich inflammatory lesions or cancers would strongly suggest that brominating oxidants formed by these cells contribute to the development of DNA damage in these disorders. Further studies are needed to unravel the role of this vs. alternative oxidation pathways, such as NO-derived oxidants, as contributors to DNA damage in breast cancer.

Personnel Supported by this Grant

DOD Breast Cancer Grant

Name	Title	Period Worked
Lambe, Kristy L.	Student	6/00-8/00
Mukhopadhyay, Chaital	Technician	1/00-5/00
Shan, Lian	Fellow	7/01-6/02
Wu, Weijia	Graduate Student/Fellow	1/00-10/00
Zhang, Renliang	Fellow	5/00-6/01

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Appendix - none